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The Discovery of Cancer Tissue Specific Proteins in Serum: Case Studies on Prostate Cancer

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1. Introduction

Carcinogenesis remains a complex and unpredictable process that involves defects in multiple signalling pathways. Environmental determinants and lifestyle practices may contribute toward their onset by the exposure to a variety of carcinogenic agents. Since the process of carcinogenesis involves the **synergistic induction** in multiple pathways inside the cell, an effective means to investigate and understand them is to engage a global approach that identifies and considers multiple changes simultaneously at the protein level (Albini & Sporn, 2007; Alderton, 2007; Hanahan & Weinberg, 2011; Mueller & Fusenig, 2004). Such an approach can be effectively engaged with the use of discovery proteomics that allows for the large-scale analysis of protein identity and expression (Anderson, Anderson, et al., 2009; Cox & Mann, 2011; Cravatt, Simon, & Yates, 2007; Diamandis, 2004; Nilsson et al., 2010; Walther & Mann, 2010; Wright, Han, & Aebersold, 2005). There is increasing strong evidence that tumorigenesis occurs in the **tissue microenvironment** as a whole, involving the active crosstalk between epithelial, endothelial, immune and stromal cells (Albini &Sporn, 2007; Alderton, 2007; Mueller & Fusenig, 2004). Consequently, the analysis at the whole tissue level is a logical initial step in the identification of tissue-specific or tissue-prevalent proteins occurring at larger concentration levels relative to those found in the systemic circulation, wherein their secretion and shedding may occur (Hanash, Pitteri, & Faca, 2008). Provided that the expressed tissue specific and prevalent proteins found in the serum or plasma represent phenotypic cancer pathophysiological events, then these proteins may be potential cancer biomarkers and/or physiologic treatment targets (Hanash, Pitteri, & Faca, 2008).

Research involving the mass spectrometry (MS) based proteomic study of fresh-frozen whole prostate tissue biopsies, cell-culture models and blood sera originating from well-defined clinical designs are discussed. Emphasis is given to those approaches involving the hyphenation of liquid chromatography with mass spectrometry by means of electrospray

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ionization (LC-MS) for the analysis of proteins derived from prostate cancer clinical specimens (S. D. Garbis et al., 2011; S. D. Garbis et al., 2008). One of the several challenges of the serum and plasma proteomic methods involve the removal of high abundant proteins (i.e. albumin, IgGs, etc.) for the in-depth analysis of the lower abundant proteins where potential biomarkers can be revealed (S. D. Garbis et al., 2011; Hanash et al., 2008). However, their removal typically results in the co-removal of a significant percentage of the lower abundant tissue specific proteins. At the same token, the co-analysis of both high and low abundance proteins and their endogenously occurring cleavage products (**serum degradome**) may confer greater insight on serum biochemistry and cancer biology. The principle themes to be covered in the present book chapter includes the development and application of quantitative bottom-up LC-MS proteomic methods in the analytical characterization of (i) fresh frozen cancerous breast and prostate tissue biopsy specimens to define proteins expressed by the tumour microenvironment, (ii) the discovery of tissue specific serum biomarkers that are secreted in the systemic circulation of clinical utility to the medical practitioner, (iii) the future perspective on the use of targeted and high-throughput LC-MS based analysis approaches for the validation of biomarker discovery findings spanning large scale specimens sets including healthy specimen cohorts], and (iv) the use of lab-on-chip formats to further enhance LC-MS analysis sensitivity, selectivity and specificity at multiple orders of magnitude lower clinical specimen amounts currently used. The analytical attributes intrinsic to these methods allow the generation of a panel of protein biomarkers with multiple molecular features as reflected on measurable analytical variables that include the chromatographic retention times indexes, the concentration level, the amino acid sequence of the proteolytic peptide, uniquely traceable or surrogate, to one particular protein, and its *in vivo* modification status. The uniqueness in molecular features encoded in a given biomaker panel is accomplished by an ensemble of analytical variables that are explicitly dependent on the collective physico-chemical properties of the proteins and their surrogate peptides that constitute this panel. The end-product from the use of such methods is the determination of tumor "signatures" at the serum or plasma level based on rationally derived protein-panels with a high degree of specificity and sensitivity that uniquely identify a particular cancer type, its stage and its applicability to personalized intervention protocols.

2. Critical elements for the study of biomarkers

Prostate cancer imposes an ever increasing healthcare burden to males worldwide due to do their higher life expectancies, the prevalence of high fat diets and sedentary lifestyles, exposure to environmental pollutants, sexual habits, etc (Albini & Sporn, 2007; De Marzo et al., 2004; DeMarzo, Nelson, Isaacs, & Epstein, 2003; Hammarsten & Hogstedt, 2002; Jemal, Siegel, Xu, & Ward, 2010; Pfeffer et al., 2002). Early detection is of vital importance in reducing mortality. However, the early detection of cancer is hampered by the lack of effective analytical methods. This lack in analytical efficiency has often resulted in the erroneous assessment and derivation of biological indicators, or biomarkers, of prostate cancer disease (Balk, Ko, & Bubley, 2003; Thompson et al., 2005). The reasons for the ineffective utility of these biomarkers are multi-fold and include the following, (i) they lack specificity and selectivity to the cancer type of interest, (ii) their reproducible detection is poor, (iii) the sensitivity of available methods, especially as they refer to biological fluids

such as serum and plasma, is poor relative to the natural abundance levels of the tissue-specific secreted or shedded molecular entities of disease, and (iv) the majority of the available analytical protocols measure biomarkers at the DNA and mRNA level, which may not reflect the phenotypic aspects of disease (Adewale et al., 2008; Buchen, 2011; Lin et al., 2005; Rahbar et al., 2011; Sawyers, 2008; Turteltaub et al., 2011). In addition, the availability of more selective prognosis strategies may also help identify patient cohorts, or even single individuals, eligible for adjuvant therapy (i.e., **personalized medicine**). Hence, new biomarkers for asymptomatic prediction, diagnosis, prognosis and response to treatment at the protein level are warranted to improve clinical intervention. It is assumed that one of the critical parameters for the staging of disease and/or treatment intervention is the difference in concentration levels found for the respective biomarkers. This especially becomes true when the complexity of the derived proteomes is decoded in the form of biological pathways and their networks that allow the interrogation of novel candidate protein markers as physiologic targets. Consequent to with this notion, the family of protein markers that will encompass the molecular biology of carcinogenesis will include not only tissue specific proteins but also proteins that reflect systemic changes that predispose a seemingly healthy individual to a longer-term initiation to event of carcinogenesis (Adewale et al., 2008; Buchen, 2011; DeMarzo et al., 2004; Hanahan & Weinberg, 2011; Joyce, 2005; Rahbar et al., 2011; Sawyers, 2008; Turteltaub et al., 2011). In addition to protein markers, and in particular enzyme species, other biological indicators of disease and its pre-disposition, may include co-factors (i.e., vitamin species) and protein end-products such as 1° and 2° metabolites in the form nucleic acids, amino acids, fatty acids and xenobiotic species in their parent and biotransformed moieties. This integrated monitoring of these biomolecular entities at multiple levels may impart more accuracy and reliability in functionally capturing biochemical pathways of disease. A general example may include an *in vivo* phosphorylation at the catalytic domain of a protein substrate leading to the inhibition of the metabolism of its affiliated ligand. The absence of biotransformed ligand constitutes a proof-positive indicator in the functional annotation of the protein under consideration. A case in point is the polymorphism of the enzyme species 5-methyl-tetrahydrofolate reductase (5-MTHFR) leading to altered concentration levels of 5-methyl tetrahydrofolate (5MTHF), a metabolically active form of folic acid. The polymorphism of 5-MTHFR has been implicated as a cause to the sub-clinical deficiency of folic acid observed in the older human adult populations, despite their adequate intake of this essential nutrient. The ability, therefore, to quantitatively monitor both the polymorphic 5-MTHFR enzyme and its biotransformation product 5-MTHF can better capture this event (Antoniades et al., 2009; S. D. Garbis, Melse-Boonstra, West, & van Breemen, 2001; Melse-Boonstra et al., 2006; Yetley et al., 2011). The unique analytical versatility and adaptability of MS based methods in detecting diverse biomolecular species imparts a unique opportunity in both customizing and validating key mechanisms of disease and its etiology. From this perspective, modulating these **mechanism based biomarkers** may cause the induction or inhibition of a given carcinogenesis pathway (Kocher & Superti-Furga, 2007). Consequently, such types of biomarkers make for better candidates as treatment targets that can be modulated with medicinal agents and other clinical intervention schemes. Our working hypothesis is based on the assumption that the key difference between the early, asymptomatic disease (low disease burden) versus that of late stage, metastatic disease (high disease burden) is the concentration level found for these mechanistic biomarkers either in their native

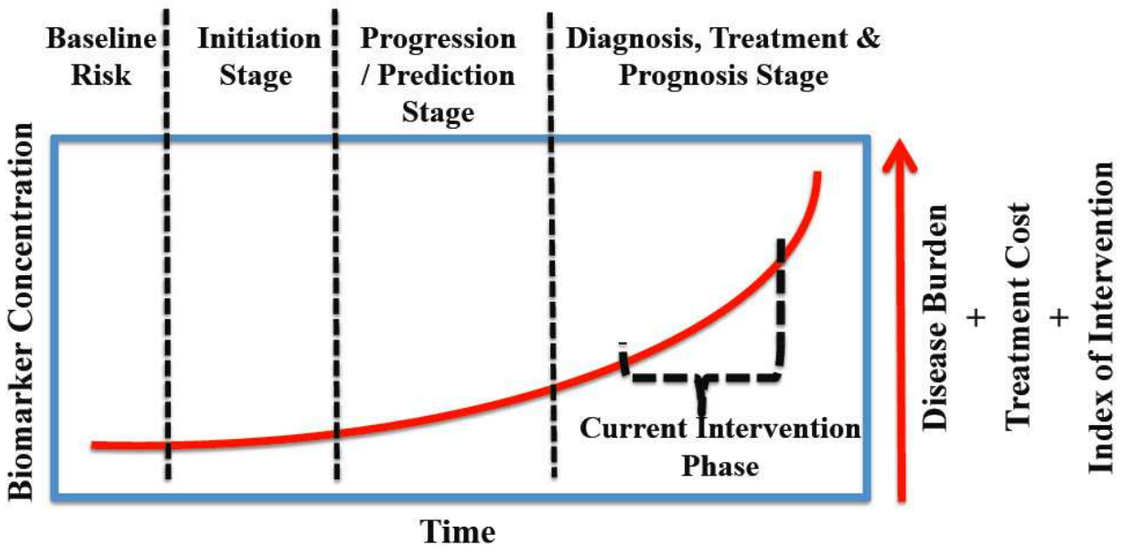

Fig. 1. A key parameter to the utility of a given biomarker is its concentration level in the assessment of disease and its treatment. The current detection methods detect biomarker levels that reflect late stage disease wherein treatment options are limited. The more sensitive and selective the analysis method the greater the effectiveness in capturing the disease progression at the initiation stage wherein its reversal is possible with cancer chemoprevention, nutritional/functional food intervention, and other low-toxicity treatment protocols.

or *in vivo* modified form (i.e., post translational modified proteins, biotransformed 1° and 2° metabolites). As such, the ability to capture very low levels of these protein markers and their surrogate end-products provides greater assurance in capturing their disease potential at the progression or even initiation stage whose effects can be reversed with less toxic intervention protocols (see Figure 1). The present discourse will focus on protein-based markers of prostate carcinogenesis.

3. Analytical chemistry background

The selection of the analytical method for the investigation of a biological specimen depends on the clinical query to be solved. However, the essential features to this selection process is for the analytical method to impart: (a) qualitative effectiveness for the identification of as many proteins as possible, (b) quantitative effectiveness to reveal absolute or relative concentrations of these proteins. The advent of mass spectrometry (MS) based techniques as compared to the other analytical techniques (i.e., molecular spectroscopy based such as fluorescence, UV-VIS, NMR; X-Ray crystallography) has allowed for the simultaneous protein identification and quantification in very small amounts of biological material, at analyte detection limits that may exceed those of fluorescence based ELISA assays as applied to clinical specimens (S. D. Garbis et al., 2011; Rubakhin, Romanova, Nemes, & Sweedler, 2011). A major milestone to the effective use of MS techniques to the analysis of a vastly larger range of biomolecules (e.g., metabolites, peptides, proteins, nucleic acids, fatty acids, steroids, etc.) was the advent of the electrospray ionization source (ESI) and its micro-

and nano- flow derivatives (Wilm, 2011). As a soft-ionization source, ESI made it possible to introduce the thermally labile biomolecular species to become introduced to the gas phase from its initial liquid phase in its charged state with an intact chemical integrity. Consequently, the ESI source allowed the interfacing of liquid phase sample introduction systems (i.e., liquid chromatography and capillary electrophoresis) with the vacuum-system encased MS platforms (i.e., quadrupolar, ion trapping, time-of-flight, or hybrids thereof, etc.). (Cox & Mann, 2011; Cravatt et al., 2007; Diamandis, 2004; Kocher & Superti-Furga, 2007; Nilsson et al., 2010; Walther & Mann, 2010). The development of novel analytical methods that are based on the combined use of liquid chromatography and tandem mass spectrometry (LC-MS) techniques for the bottom-up or top-down proteome analysis of a wide spectrum of both low and high abundant proteins in clinical tissue and sera dates back to the late nineties with the introduction of the Multi-Dimensional Protein Identification Technology (MudPIT) by John Yates (Fournier, Gilmore, Martin-Brown, & Washburn, 2007). The MuDPIT approaches constituted an alternative to the Two-Dimensional Gel Electrophoresis (2DGe) approaches in their ability to capture and identify a wider spectrum of proteins and at lower abundance levels. These in-depth LC-MS proteomic methods employ the orthogonal use of various high-performance liquid chromatographic (HPLC) chemistries, based on the principles of strong ion exchange (XIC), size-exclusion (SEC), hydrophilic interaction (HILIC), affinity capture (biological and chemical), reverse phase (RPC) and others. These separation techniques allow the isolation, separation and enrichment of proteins and surrogate peptides found in extracts derived form clinical specimens such as tissues, blood plasma and sera. Overall, the LC-MS proteomic methods incorporate the combined use of both nano-electrospray ionization (nESI) and off-line matrix-assisted laser desorption ionization (MALDI) interfaces, to ensure the broadest possible surrogate peptide coverage for a given protein. The bottom-up analysis approach, which is based on the analysis of surrogate tryptic peptides, is well suited for a robust and sensitive protein analysis strategy (taking into consideration individual protein hydrophobicity, charge, or post-translational modification). These complementary methodological approaches provide a more comprehensive and reproducible proteomics assessment of clinical tissue and sera specimens. This has become yet more evident with the use of the latest tandem MS-MS analyzer platforms that include the quadrupole time-of-flight QqTOF and Orbitrap based geometries. These MS platforms exhibit high-sensitivity (limit of detection < 10 fmol on-column allowing the use of very low signal accumulation times) and ultra-high resolution (≥ 30,000, translating to 1-3 ppm mass accuracies) at very high signal sampling speeds (≥ 30 Hz). Such performance characteristics allow the detection > 3,000 proteins at > 99% confidence derived from cell culture lysates and spanning over 4-orders of magnitude natural concentration abundance in a single LC-MS analysis run (Cox & Mann, 2011; Liu, Belov, Jaitly, Qian, & Smith, 2007; Mann & Kelleher, 2008; Ong & Mann, 2005). One of several key advantages of the non-gel LC-MS based methods is that they allow the analysis of a much wider spectrum of proteins than that typically covered with the classical gel-based approaches. This spectrum includes proteins that are membrane bound or membrane associated; proteins that exhibit alkaline (pI > 8) and acidic (pI < 5) character; proteins with low (<10 kDa) or high (>200 kDa) molecular weights; and proteins that have undergone *in vivo* modifications (i.e. phosphorylation, acetylation, methylation, glycosylation, etc.) occurring in minor molar ratios (oftentimes < 1:1000) relative to their

native counterparts (S. Garbis, Lubec, & Fountoulakis, 2005; Lubec & Afjehi-Sadat, 2007; Nilsson et al., 2010; van Bentem, Mentzen, de la Fuente, & Hirt, 2008). Currently more than 150 different types of *in vivo* modifications are possible (Seymour et al., 2006; Shilov et al., 2007). The ability to detect and discriminate these post-translational modified proteins constitutes a major advancement in the more comprehensive understanding of signaling cascades at the protein level allowing for a more direct appreciation of protein-protein interaction and consequently biological pathways and their networks (Kocher & Superti-Furga, 2007; Mann & Kelleher, 2008; Ong & Mann, 2005; van Bentem et al., 2008). It is assumed that the vast majority of proteins have undergone multiple and diverse *in vivo* modifications that define their induction or silencing status. Such protein traits can only be captured with tandem MS spectra generated at high sensitivity and high resolution providing unequivocal evidence in the annotation of their *in vivo* modification at the precise amino acid location in single LC-MS experiment (Liu et al., 2007; Mann & Kelleher, 2008; Ong & Mann, 2005; Papayannopoulos, 1995). Conceptually, a vast array of *in vivo* modifications can be captured and stored for later use as means to provide a multifactorial understanding of biological pathways and their networks. The current biochemical assays such as Immunohistochemistry and Western blots fail to account for these intrinsic protein *in vivo* modification traits. It is this limitation that has often resulted in the analysis bias between the MS and biochemical assay measurements (Diamandis, 2004; Lubec & Afjehi-Sadat, 2007; Nilsson et al., 2010).

The collective LC-MS analysis characteristics constitute a major advancement toward an in-depth proteome analysis of the fresh-frozen tumor specimens.Advanced proteomics approaches can bridge the gap between the genetic and epigenetic alterations underlying cancer and cellular physiology. The precepts of multidimensional liquid chromatography hyphenated with high resolution, tandem mass spectrometry (MDLC-MS-MS) techniques in combination with the use of isobaric tags for relative and absolute quantification (iTRAQ™) of whole tissue biopsies of various types of cancer tissue (i.e., breast, prostate, cervical) has played a key role in bridging this gap. In general, a key advantage of 2DLC-MS-MS methods that utilize isobaric stable isotope based approaches (i.e., cICAT, TMT, iTRAQ, etc.) is the ability to conduct multiplex experiments, whereby specimen extracts can be analyzed concurrently under the same experimental conditions. This multiplexing advantage reduces systematic error, and improves the signal-to-noise of the precursor MS and product ion MS-MS response allowing for a greater number of proteins to be quantitatively profiled (DeSouza et al., 2005; S. D. Garbis et al., 2008; Glen et al., 2008; Pichler et al., 2011; Wu, Wang, Baek, & Shen, 2006). Advancements made to liquid chromatography and mass spectrometry stand to further potentiate the utility of these isobaric stable isotope tags (Fournier et al., 2007; Pichler et al., 2011). Other key attributes that make MS based methods the premier choice for the analysis of small amounts of clinically valuable and complex biological specimens along with reduced requirements for stable isotope reagents is driven by the increased automation and miniaturization imparted by lab-on-a-chip formats (Everley, Krijgsveld, Zetter, & Gygi, 2004; Koster & Verpoorte, 2007; Rubakhin et al., 2011; Tsougeni et al., 2011). These themes are covered within the context of case studies in the analysis of clinical whole tissue biopsies and their sera for prostate cancer.

4. Prostate cancer

4.1 The quantitative proteomic profiling of clinical whole tissue biopsies derived from benign prostate hyperplasia and prostate cancer

Prostate whole tissue biopsies exhibit extensive biological variability when accounting for the diversity in human subjects and the heterogeneity and size of the tissue specimen itself. These variables must be taken into consideration when executing its proteomic study. Factors such as tissue procurement, histopathology pre-assessment, storage, handling, and pre-analytical processing, and instrumental performance verification with standardization (chromatographic and nano-ESI ionization efficiency, MS and MS-MS sensitivity, resolution, accuracy and precision) are variables that need to be optimized for any given proteomic study. The optimization of these variables will minimize the histopathological, biological, pre-analytical and analytical variability so essential to a reproducible and information-rich proteomic output (Buchen, 2011; Cox & Mann, 2011; Diamandis, 2004; Hilario & Kalousis, 2008; Nilsson et al., 2010).

Several multiplex proteomics studies that rely on the use of cysteine-specific isotope-coded affinity tags (cICAT), stable isotope labeling with amino acids in cell culture (SILAC), difference gel electrophoresis (DIGE) and trypsin-mediated ^{18}O isotope labeling have been successful in detecting differentially expressed proteins in combined specimen samples (DeSouza et al., 2005; Everley et al., 2004; Hood et al., 2005). Despite their advantages however, intrinsic limitations exist for each of these approaches. The cICAT approach allows only the labeling of proteins containing cysteine residues on tractable peptides upon proteolysis making this approach unsuitable as a comprehensive and in-depth protein discovery tool. The cICAT approach has been used for the quantitative proteomic profiling in secondary prostate cancer cell cultures. In one such study, 524 secreted proteins were from the LNCaP neoplastic prostate epithelium of which 9% of these were found to be differentially expressed (Martin et al., 2004). In another study involving the same cell culture model in response to androgen exposure resulted in the identification of 1064 proteins of which approx. 21% of these proteins were modulated (Wright et al., 2004).

Another label-based approach for prostate biomarker discovery efforts makes use of heavy water. In such an approach, $H_2{}^{18}O$ water is used instead of regular water for the solution phase trypsinization process thus allowing the trypsin-mediated ^{18}O stable isotope incorporation (^{18}O labeling) for those proteins extracted from one specimen category (i.e. control, treated or diseased states). This process leads to the exchange of two equivalents of ^{16}O with two equivalents of the ^{18}O stable isotope at the carboxyl terminus of the resulting tryptic peptides coined as the «heavy» peptides. The heavy water approach was applied to proteins extracted from benign prostate hyperplasia (BPH) vs. prostate cancer (PCa) cells isolated from a single formalin-fixed paraffin embedded (FFPE) prostate cancer tissue specimen (Hood et al., 2005). This study resulted in the quantitative profiling of only 68 proteins. The limited proteins amounts along with their cross-linked form limit the utility of FFPE as a viable specimen source for proteomic assessment. Another confounding factor in the practical utility of the ^{18}O labeling strategy, which also applies in cICAT labeling case, is that only two samples can be analyzed per experiment.

A gel-based relative quantitative approach that has been used for prostate cancer cells is known as the differential gel-electrophoresis (DIGE). The DIGE method represents a variant

of the classical 2-D gel electrophoresis (2DGE) technique whereby CyDye fluorescence probes are used as tags to covalently modify proteins without affecting their electrophoretic properties. Consequently, the resulting CyDye fluorescence labeled proteins originating from multiple biological specimens migrate to almost the same location of a 2-D gel. Using this approach, up to three different fluor labeled samples can be combined and 2DGE separated in a single experiment thus allowing better spot matching and reduction in gel-to-gel non-reproducibility. One fundamental drawback to the DIGE approach is its MS-incompatibility because of the ionization suppression effects induced by fluor labeled reagents. Consequently, all the intrinsic gel-based limitations also apply for the DIGE approach (S.Garbis et al., 2005; Garcia-Ramirez et al., 2007; Lubec & Afjehi-Sadat, 2007; Wu et al., 2006). The use of the DIGE based method was applied to the study of perturbed protein networks in LNCaP prostate cancer cells administered to both androgen and anti-androgen exposure resulting in the quantitative profiling of 107 proteins (Rowland et al., 2004).

The development and application of a quantitative proteomic method involving the use of off-line size-exclusion chromatography (SCX) followed by the on-line reverse phase (RP) chromatography hyphenated with high resolution, tandem mass spectrometry (2DLC-MS-MS) in combination with the use of isobaric tags for relative and absolute quantification (iTRAQ™) was applied to the analysis of clinical whole tissue biopsies derived from patients with benign prostate hyperplasia (BPH, n=10) and prostate cancer (PCa, n=10)(S. D. Garbis et al., 2008). Key advantages to this approach include the ability to conduct multiplex experiments, whereby up to eight samples can be analyzed concurrently under the same 2DLC-MS conditions, resulting in reduced systematic error and increased electrospray ionization efficiency leading to higher sensitivity; in addition, since protein identification and quantification is based on tandem mass spectrometric (MS-MS) evidence, increased selectivity, specificity and confirmatory power are achieved. This study resulted in the reproducible quantitative profiling of 827 proteins of which 65 were differentially expressed. The access to well defined human whole prostate tissue biopsies allowed for the investigation of the stromal vs. epithelial cell interaction in the manifestation of prostate cancer. An essential requirement to the iTRAQ 2DLC-MS-MS approach is the use effective liquid chromatographic technique to impart sufficient separation of the large number of tryptic peptides generated. This will reduce the co-eluting peptides that would otherwise result in erroneous product ion MS-MS spectra negating the accurate relative quantification efficiency and protein identification accuracy (Fournier et al., 2007). The modulated proteins identified were implicated in the inflammation response (Albini et al., 2007; Albini, Tosetti, Benelli, & Noonan, 2005; DeSouza et al., 2005; Goldstraw, Fitzpatrick, & Kirby, 2007; Nelson, DeMarzo, DeWeese, & Isaacs, 2005), the modulation of the androgen (Cheung-Flynn et al., 2005; De Leon et al., 2011; Hildenbrand et al., 2011; McKeen et al., 2011; Milad et al., 1995; Miyoshi et al., 2003; Nelson et al., 2005; M. H. Yang & Sytkowski, 1998), and prostate cancer metastasis (Ablin, Kynaston, Mason, & Jiang, 2011; Dabbous, Jefferson, Haney, & Thomas, 2011; Di Cristofano et al., 2010; Grisendi, Mecucci, Falini, & Pandolfi, 2006; Hale, Price, Sanchez, Demark-Wahnefried, & Madden, 2001; Jiang & Ablin, 2011; Khanna et al., 2004; C. J. Kim, Sakamoto, Tambe, & Inoue, 2011; Krust, El Khoury, Nondier, Soundaramourty, & Hovanessian, 2011; Moretti et al., 2011; Okuda et al., 2000; Planche et al., 2011; Sun, Song, et al., 2011; Sun, Zhao, et al., 2011; Weng, Ahlen, Astrom, Lui, & Larsson, 2005; Yu & Luo,

2006), as essential hallmark features for these prostate cancer tissue specimens. Another interesting finding that also goes toward validating the accuracy of the proteomic method is the differential expression of several prostate specific cancer markers such as the prostate-specific transglutaminase, the prostate associated gene 4 protein, the prostatic acid phosphatase, and the prostate specific membrane antigen (see Figure 2). The presence of the prostate-specific transglutaminase in PCa has been recently reported as a potential anti-tumour target (Ablin et al., 2011; Jiang & Ablin, 2011). Yet another important finding from this study were proteins reported to be implicated as potential cancer chemoprevention targets also affiliated with poor nutritional status and metabolic syndrome disease (Das et al., 2011; De Nunzio et al., 2011; DeMarzo et al., 2003; Dong, Zhang, Hawthorn, Ganther, & Ip, 2003; Gonzalez-Moreno et al., 2011; Jeronimo et al., 2004; J. Kim et al., 2005; Kuemmerle et al., 2011; Menendez & Lupu, 2007; Nelson et al., 2005; Oh et al., 2006; Sytkowski, Gao, Feldman, & Chen, 2005; Toki et al., 2010; Tsavachidou et al., 2009; Walsh, 2010; C. M. Yang, Yen, Huang, & Hu, 2011; Zeliadt & Ramsey, 2010). These proteins include the retinol binding protein I, selenium binding protein 1, fatty acid synthase, and insulin-regulated lipase and are oftentimes synergistically expressed with other proteins implicated in the inflammation response and androgen regulation.

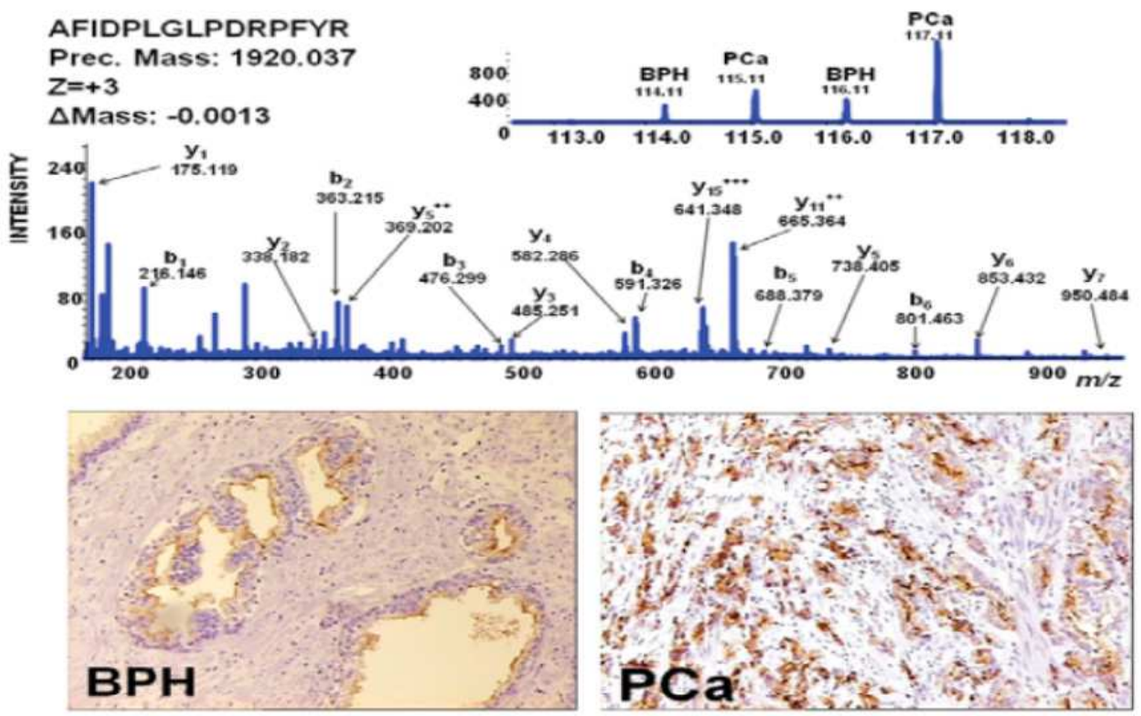

Fig. 2. A surrogate peptide sequence and its relative quantification indicating the over expression of prostate-specific membrane antigen (PSMA) in prostate cancer (PCa) vs. benign prostate hyperplasia (BPH) with corresponding immunohistochemical confirmation for these specimen categories that effectively corroborate the quantitative proteomic findings (S. D. Garbis et al., 2008).

4.2 The quantitative proteomic profiling of clinical serum samples derived from benign prostate hyperplasia

Tissue proteomics is considered a logical first step for the novel discovery of tumour-derived proteins as they exist in higher concentrations due to their more direct proximity to cancer cells (Cravatt et al., 2007; Hanash et al., 2008; Joyce, 2005; Mueller & Fusenig, 2004; Wright et al., 2005). However, it is not well understood how protein expression in tissues reflect measurable levels in the serum or plasma that would allow the monitoring of the pathophysiological status of respective tissue (Anderson, 2010; Barelli, Crettaz, Thadikkaran, Rubin, & Tissot, 2007; Farrah et al., 2011; Hanash et al., 2008; Issaq, Xiao, & Veenstra, 2007). This may partially stem from the trend that the comprehensive analysis of tissue relevant proteins in less invasive clinical matrices such as the plasma or serum has been a daunting task for MS based methods despite all their latest technological advancements (Anderson, 2010; Farrah et al., 2011; Hanash et al., 2008). For example, currently available serum and plasma proteomics methods rely on the prior removal of high abundant proteins (i.e. albumin, IgGs, etc.) so that the lower abundant proteins, where potential biomarkers can be revealed, could be more easily analyzed. Several studies, however, have shown that their removal also resulted in the co-removal of a significant percentage of these lower abundant proteins due to their propensity to bind with the higher abundant proteins (S. D. Garbis et al., 2011; Granger, Siddiqui, Copeland, & Remick, 2005; Gundry, White, Nogee, Tchernyshyov, & Van Eyk, 2009; Zolotarjova et al., 2005). Additionally, these studies correctly purport than no MS based method to date has managed to fully remove albumin and other high abundant proteins despite claims made on the contrary. It is estimated that the 20 most abundant proteins in serum and plasma constitute over 99% of the total protein mass found in these matrices. In fact, the difference in endogenous concentration levels of proteins found in serum or plasma span from the mg/mL level (i.e. Albumin, IgG's) down to the low ng/mL level (i.e. Cyclin F, Interleukin 7) (Anderson, 2010; Farrah et al., 2011). This represents a 12-order of magnitude concentration range whose lower limit exceeds the detection capability of the fluorescence based ELISA technique, the most sensitive bioassay technique to date (Rissin et al., 2010). At the same token, the detection of endogenously occurring cleavage products (serum degradome) originating from both high and low abundance proteins may confer greater insight on serum biochemistry and cancer biology (van Winden et al., 2010). This is considered a very important incentive for the whole proteome wide analysis of the serum or plasma matrix in the prospecting of mechanism based biomarker panels.

In an effort to overcome these challenges, an approach coined multidimensional protein identification technology (MudPIT) has been developed (Fournier et al., 2007; S. D. Garbis et al., 2011; Hanash et al., 2008). This approach is principally based on combining two or more different types of liquid chromatographic chemistries so as to increase the separation efficiency as a result. This effect on the separation power is referred to as "orthogonal chromatography" and constitutes a very unique and powerful tool towards the more effective analysis of complex biological matrices (cell cultures, tissues, serum and plasma). Building on this theme, a three-dimensional (3-D) MudPIT variant was developed and applied to the analysis of clinical sera derived from patients with (BPH). The tissues from these BPH patients were analyzed and reported with the iTRAQ 2DLC-MS discussed in the previous section and was considered requisite for this proof-of-principle study so as to

explore the possibility of finding tissue specific proteins in their respective serum (Garbis et al, 2011).

The analytical features of the 3-D MudPIT approach included (Figure 3): (1) high pressure size-exclusion chromatography (SEC) for the pre-fractionation of serum proteins followed by their dialysis exchange and solution phase trypsin proteolysis, (2) The tryptic peptides were then subjected to offline zwitterion-ion hydrophilic interaction chromatography (ZIC-HILIC) fractionation, and (3) their online analysis with reversed-phase nano ultra-performance chromatography (RP nUPLC) hyphenated to nano-electrospray ionization - tandem mass spectrometry.This orthogonal chromatographic strategy used imparts a more effective parsing, purification and enrichment of the tryptic peptides when combined with the prior SEC protein pre-fractionation stage. This has the effect on increasing their individual mass density of the tryptic peptides (higher peptide signal intensity per

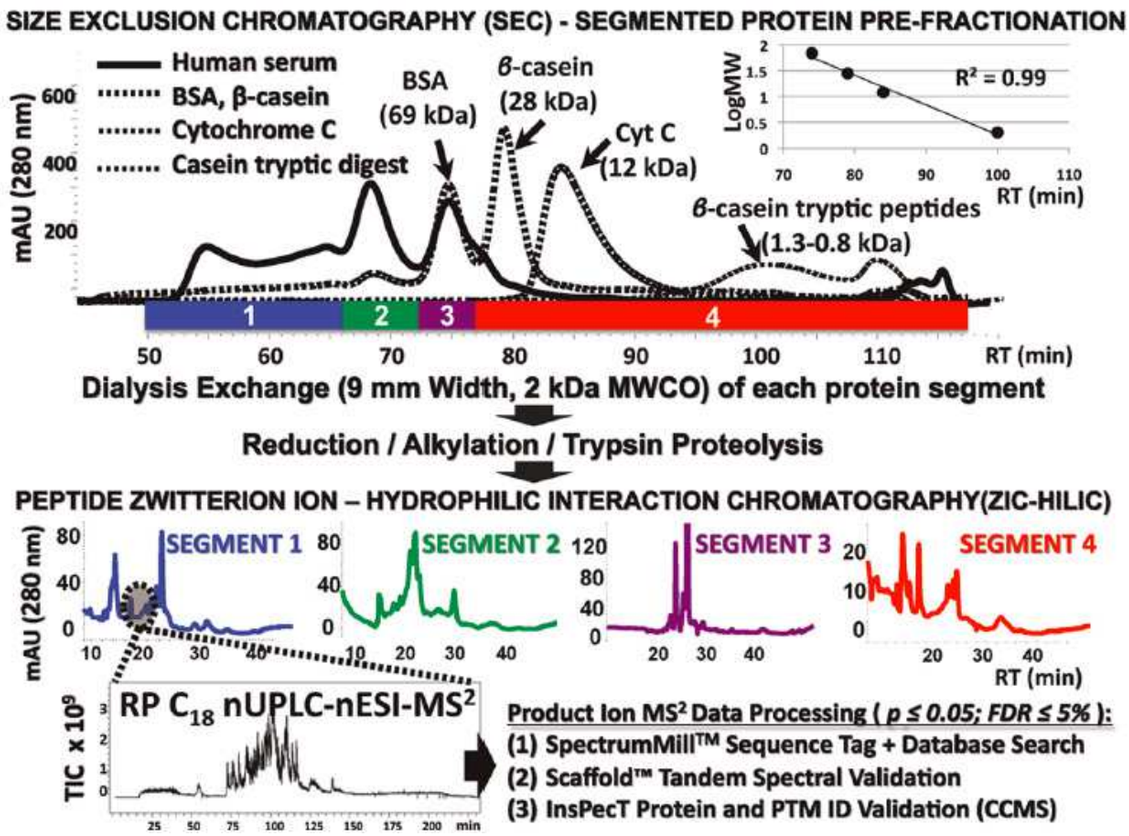

Fig. 3. **Top HPLC trace:** A representative size exclusion chromatography (SEC) trace of a pooled serum sample. Calibrant SEC traces are also shown along with their log MW vs. RT (min) linear response curve. **Middle HPLC traces:** Post-SEC sample treatment and ZIC HILIC tryptic peptide traces in concordance to SEC protein segment. The ZIC-HILIC peptide fractionation was performed in a peak-dependant manner. **Bottom HPLC trace:** Each lyophilized peptide fraction was reconstituted in MP and individually analyzed with RP C_{18} nUPLC-nESI-MS^2 analysis. The resulting product ion MS^2 peptide spectra were processed with Scaffold™ validation, SpectrumMill™ and InsPecT software programs (Garbis et al, 2011)

chromatographic retention time window) while at the same time reducing their co-elution (improved separation efficiency). It is precisely these chromatographic characteristics that allowed the enhancement of the nano-electrospray ionization of the eluting peptides followed by their tandem mass spectrometry. The end result from this process was the generation of more information rich tandem mass spectra at improved S/N ratios, which constitutes the ultimate objective for any effective MS based method.

Consequently, the collective analytical attributes of this milestone 3-D MudPIT analysis study of BPH sera resulted in the identification of proteins differing by approximately 12-orders concentration range in terms of their native abundance levels in the naturally occurring serum matrix (as measured with bioassay technique such as ELISA). In addition to this extensive dynamic range coverage, the study identified 1955 proteins with a wide spectrum of biological and physico-chemical properties. A key component however to this proteome including the detection of secreted, tissue-specific proteins also found to be differentially expressed in the respective BPH tissue reported (S. D. Garbis et al., 2008). This constitutes a hallmark feature in the effective discovery of serum protein markers that reflect the pathophysiology of a specific organ tissue of interest. An additional performance characteristic of the 3-D study method is its accuracy and sensitivity in identifying close to 400 phosphoproteins of potential importance to cancer biology. The identification of the phosphorylated variant to a potential protein marker imparts an additional molecular feature in the more precise capturing of unique chemical signatures of disease. This is based on the notion that a phosphorylated motif may signify the induction or silencing of a potential physiologic protein target already discussed. The versatility and adaptability of the method's constituent techniques permit the incorporation of label-based or label-free strategies to impart a quantitative feature for the in-depth proteome analysis of any given biological specimen derived from tissue, blood plasma or serum, and cell culture.

The tissue-surrogate serum proteins detected in this study and other MudPIT studies allow for the un-biased and in-depth discovery of useful biomarkers without recourse to the targeted antibody capture approach, as is common the case. In contrast, the Medical Therapy of Prostatic Symptoms (MTOPS) clinical trial, attempted to characterize potential biomarkers that could stratify the BPH patients according to their response to medical therapy, by using the *a priori* use of the ELISA assay (Mullins et al., 2008). However, such an *a priori* approach bypassed the possibility in observing unexpected low-abundant tissue specific and secreted proteins that might play a significant role on the differential diagnosis between BPH and PCa. Conclusively, the MudPIT approach is definitely a forward trend in the establishment of novel proteins marker that can be validated with more targeted approaches such as those based on Immuno-MRM techniques discussed below.

5. Future trends

5.1 Immuno-SRM (SISCAPA)

The comprehensive qualitative protein identification capability of the MudPIT approaches, can be extended to include relative quantitative features made possible with the use of multiplex stable isotope labelling strategies at the protein or peptide level. As already discussed, the quantitative capability will further minimize analytical systematic error and to better stratify patients in accordance to prostate pathophysiology analogous to that of the

BPH/PCa prostate tissue study reported by the authors. Such an approach can serve as part of a more systematic serum biomarker discovery study that can eventually lead to their validation over a very large number of specimens from healthy and diseased patient cohorts, typically exceeding 1000 for each group. So far, however, and despite the advancements made in analytical technologies, the discovery and validation of robust protein biomarkers with good specificity and sensitivity has been very disappointing. This low return on investment is due to several factors. One of them is due to the lack of functional or mechanistic utility of the candidate biomarkers. This lack of mechanistic relevance also applies to proteins that exhibit a significant differential expression between the healthy and disease samples. Another factor is associated with the large biological heterogeneity of the specimens tested. Unless the clinical samples have well defined inclusion and inclusion criteria along with effective sample procurement and handling protocols at statistically significant numbers to address a hypothesis at hand (i.e., power analysis), the analytical output will lack accuracy and precision to be of any value to the clinician (Adewale et al., 2008; Anderson, 2010; Barelli et al., 2007; Farrah et al., 2011). Another impediment is the lack of lower-cost and high-throughput validation protocols to compensate for the large number of samples that need to be analyzed. This is further compounded by the lack of antibodies for the vast majority of candidate proteins needed for the development of an ELISA kit, which is the only suitable bioassay for protein measurements in serum or plasma. Yet another limitation relates to the unreliability of a significant number of commercially available ELISA kits due to their lack of sufficient antibody validation in terms of their selectivity, cross-reactivity, linear dynamic range and sensitivity(Bordeaux et al., 2010; Stoevesandt & Taussig, 2007). An additional factor to the high failure rate of the effectiveness of the ELISA assay is that its development is principally based on recombinant protein standards that do not capture the level of complexity of the protein as it exists its *in vivo* modification status within the context of its biological matrix and also the level of protein purification is not high enough to compare to the behavior observed for the respective recombinant, highly purified, protein. Moreover, the ELISA assay is not conducive to multiplexing approaches that could have reduced some of the biological variation already discussed. This is where targeted tandem mass spectrometry methods can overcome these limitations (Gerber, Rush, Stemman, Kirschner, & Gygi, 2003; Jaffe et al., 2008). Examples of these methods include accurate inclusion mass spectrometry (AIMS) and quantitative selection reaction monitoring (Q-SRM). These more targeted MS methods specifically account for the amino-acid composition of surrogate tryptic peptides to which the selective monitoring of their precursor mass (i.e., with quadrupole mass filter), its fragmentation (i.e., CID, HCD, ETD), and subsequent product ions take place. This Selective towards one specific peptide MS precursor – product ion Reaction Monitoring (hence the term SRM) allows for its more full-time measurement and henceforth its enhanced detection in complex mixtures. The SRM detection is therefore based on the molecular signature (i.e. the unique amino acid composition of a peptide) traceable to an information rich, distinctively annotatable (i.e., *de novo* peptide sequencing), tandem (MS-MS) spectrum. Also, the intensity of the tandem spectrum traceable to one specific peptide depends on the relative or absolute concentration level of this peptide (Q-SRM). Such a level of selectivity and specificity is well beyond what can be attained with antibody capture technologies (i.e., ELISA assay)(Rissin et al., 2010). In addition, the detection of a biochemical assay is based on an absorption reading to a specific wavelength that is highly subject to background signal

interference due to cross-reactivity or non-specific binding effects. Another innate advantage to the SRM technique is their very large linear dynamic range that exceed 4-orders of magnitude thanks to the latest developments to MS analyzer and detector technology (Cox & Mann, 2011; Nilsson et al., 2010; Zhang et al., 2011). When one accounts

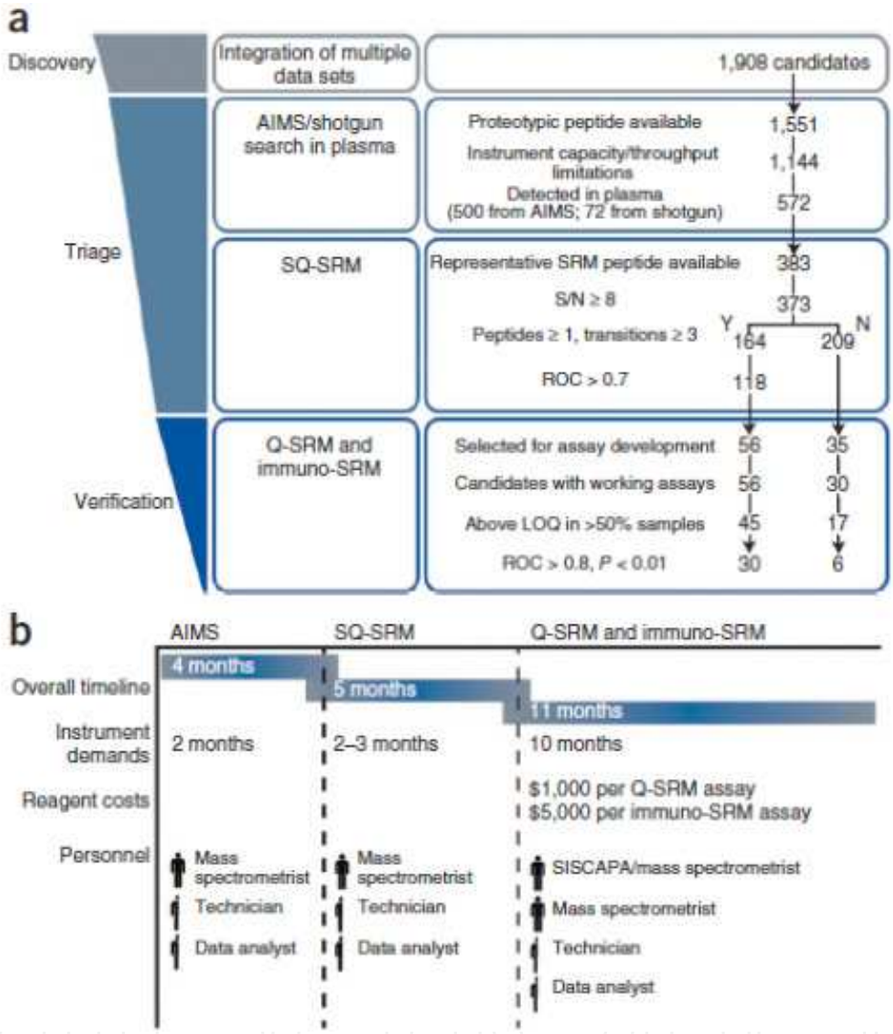

Fig. 4. Multistage, targeted proteomic pipeline for triage and verification of biomarker candidates. (a) Overview of the workflow used to triage and verify candidate biomarkers, showing the flux of candidates at each stage of the pipeline. (b) Required resources forimplementing the proteomic pipeline. The overall timeline includes time for data collection and analysis. For Q-SRM and immuno-SRM measurements, the overall timeline includes synthetic peptide quality control, development of SRM methods, acquisition of response curves and data analysis (but not the time required to generate antibodies, which can be interspersed with other activities). Instrument demands are summarized independently to provide an estimate of the required laboratory resources to carry out the study. Additional reagent costs (e.g., peptide standards and antibodies) are required for Q-SRM and immuno-SRM assays. Finally, the required personnel used in each phase of the study are denoted as full-time equivalents (Whiteaker, J.R., et al., *Nat. Biotech.* 2011).

for both the enhanced measurement selectivity and dynamic range characteristics, the SRM technique can attain > 1-2 orders of magnitude greater sensitivity compared to the fluorescence ELISA assay. These SRM advantages can be extended when combined with various targeted protein or tryptic peptide isolation techniques such as biological antibody capture (i.e., monoclonal or polyclonal based Immuno-SRM) or chemical affinity capture (i.e. chemical ligands, peptide aptamers, etc.). In the case of the Immuno-SRM variant, it can be tailored to accommodate polyclonal antibodies for the immunoaffinity capture and enrichment of proteotypic peptides mixed with their stable isotope analogs as internal standards upstream to the SRM-MS detection phase. In this scenario, the proteins found in biological extracts are tryptic digested, the resulting tryptic peptides are then immunoaffinity isolated with the polyclonal antibodies, mixed with the specific stable isotope analogues and then analyzed with SRM-MS techniques. The stable isotope peptide analogues are used as internal standards to allow for absolute or relative quantification. This particular work-flow is referred to as Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA)(Anderson, Jackson, et al.,2009; Whiteaker et al., 2011; Whiteaker, Zhao, Anderson, & Paulovich, 2010). The principles of the SISCAPA – SRM MS work-flow allows for the simultaneous capturing and analysis of over 50 tryptic peptides, uniquely traceable to over 10 proteins (Kuzyk et al., 2009). With proper experimental design and the employment of effectively trained analysts, the SISCAPA has great potential in the high-throughput with high-confidence (> 90%) analysis of thousands of clinical serum or plasma specimens for the reliable verification and validation of protein biomarker panels as unique signatures of disease prediction, diagnosis, or treatment prognosis (Figure 6). The high analysis capacity afforded by the SISCAPA - SRM MS workflow can permit the implementation of double blind, randomized and placebo controlled clinical designs (i.e., to also include a statistically significant number of healthy volunteer with diseased patient cohorts) for the more robust and comprehensive validation of such biomarker panels. Another potential advancement achievable with the SISCAPA - SRM MS workflow can be for to supersede the other currently available protein verification assays such as the Western blot, qRT-PCR, protein chip arrays, etc. This is based on the notion that workflows such as that of the SISCAPA - SRM MS can match the selectivity, specificity and sensitivity achievable by the high-precision discovery MS methods such as those based on the 3-D MudPIT and iTRAQ 2DLC-MS techniques already discussed. This especially becomes true when the same tryptic peptides including those that have undergone *in vivo* modification constitute the analytes to be measured.

5.2 Microfluidics and Lab-on-a-chip

A crucial requirement in the ability to study the content of a biomedical specimen such as clinical tissue biopsies, cell cultures or blood plasma/serum for the presence of potentially significant biomarkers is analytical sensitivity. This especially becomes prudent when the staring amount of a given clinical specimen is small. Additionally, sensitivity becomes absolutely essential given that the concentration of clinically relevant proteins and their surrogate biomolecules is exceedingly small at the progression or initiation stages of carcinogenesis already discussed. When combined with selectivity, that is an affinity to preferentially analyze one specific biomolecular entity, the availability of high sensitivity allows the targeted analysis of a naturally low abundant disease marker in complex matrices such as those typically encountered in clinical specimens. It is these requirements that drive

the advancements made in microfluidic lab-on-chip (lab chip) devices (Astorga-Wells,Vollmer, Bergman, & Jornvall, 2005; Culbertson, 2006; Gottschlich, Culbertson, McKnight, Jacobson, & Ramsey, 2000; Koster & Verpoorte, 2007; Lion et al., 2003). The ever more effective bioanalyte detection is driven by the ability to integrate their extraction from complex multi-cellular matrices at decreased dilutional effects, followed by their ultra high-resolution separation, enrichment and purification upstream to the MS detection process. The lab chip devices actualize such a principle. One of several optimal characteristics of a lab chip device includes the high surface-to-volume (S/V) ratios of its microfluidic channels for analyte capture and chromatographic separation. Maintaining optimum S/V ratios is conducive toward fast and effective interaction between the solution phase bioanalyte with the stationary phase binding site. As a result, the bioanalyte to be measured exhibits

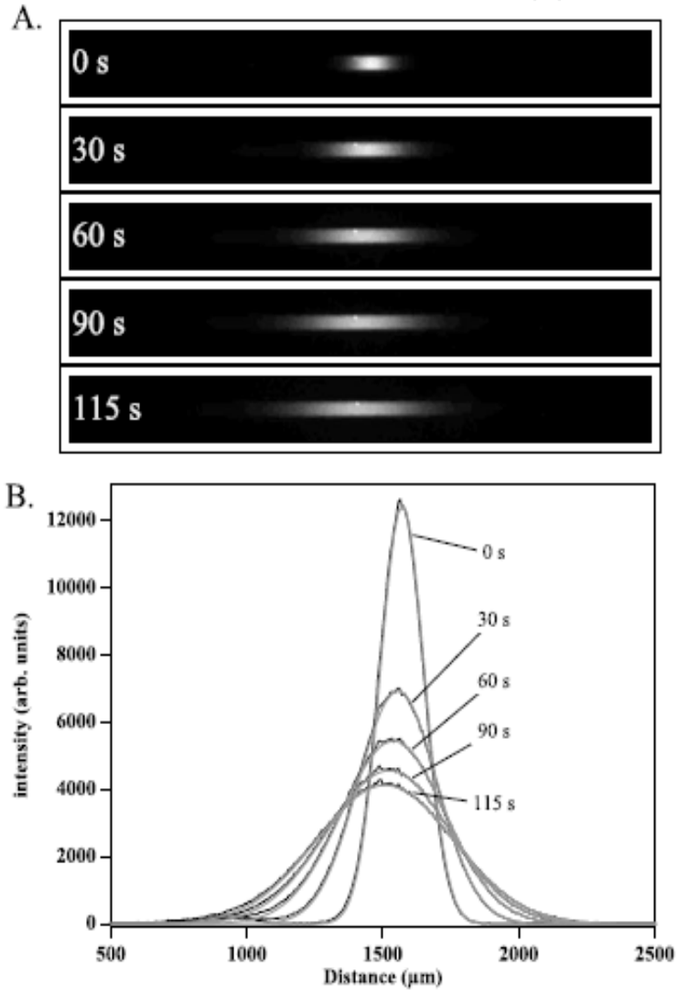

Fig. 5. Illustration of the lateral-diffusion effect of a specific amount for a given analyte species under a constant chromatographic medium. The analyte amount gets distributed over a wider distribution due to its diffusion in a time dependent manner therefore reducing its mass density at the apex. The decrease of the diffusional path of the analyte results to the increase of its mass density at the apex and consequently its improved detection at this point (Culbertson et al., 2002).

decreased lateral diffusion thus increasing its mass density leading to improved MS-based measurement sensitivity (Figure 7)(Culbertson, Jacobson, & Michael Ramsey, 2002). Other physico-chemical parameters that also play a role in achieving ideal diffusional kinetic profiles for a given chromatographic process, include the geometry of the chambers, their material properties (i.e., porous vs. non-porous), the actual chemistry (i.e. ion-exchange, hydrophilic/hydrophobic interaction, etc.) and configuration (packed vs. open tubular) of the interactive binding sites, the chemical composition of the solution phase (i.e., affecting viscosity, ion charge and mobility, etc.)(Culbertson et al., 2005; Koster & Verpoorte, 2007). Concordantly, the lab chip devices can fully exploit the very high-speed (> 40 kHz) with high-resolution (>30,000 m/Δm) signal acquisition features of the current MS platforms retrofitted with on-line or off-line ionization interfaces, such as the ESI or MALDI type, respectively.

These design features were incorporated in the development and application of a lab chip device based on a TiO_2–ZrO_2 monolithic chemical affinity chromatography format for the more selective and sensitive analysis of phosphopeptides at higher loading capacities relative to other more mainstream approaches such as those based on micropipette tips (Tsougeni et al., 2011). This monolithic column was configured on 2 mm PMMA plates, and consisted of 32 parallel microchannels with common input and output ports (Figure 8). The isolated, purified and enriched phosphopeptides were deposited onto a MALDI target and then off-line analyzed with a MALDI-MS system. The phosphopeptide binding specificity of the bidentate TiO_2–ZrO_2 chemistry at acidic pH environments, the larger number of theoretical plates (or, the density of these binding sites per unit area), and the high S/V ratio of microporous monolithic configuration all corroborated towards achieving this goal.

Conceptually, multiple chromatographic modalities can be integrated in a single lab chip format thanks to the latest developments of piezo-electric actuators, cantilevers, micro-pumps and valves, micro- and nano- mixing chambers, electroosmotically induced hydraulic pumping and other lab chip components (Figure 9). As such, these components operate under very small flow-rates (1-10 nL/min) conducive toward the optimum operation of nano-chromatographic dimensions (i.e. inner diameters < 20 μm) that also incorporate the attributes just discussed (Culbertson, Ramsey, & Ramsey, 2000; Hoeman, Lange, Roman, Higgins, & Culbertson, 2009; Jahnisch, Hessel, Lowe, & Baerns, 2004; McKnight, Culbertson, Jacobson, & Ramsey, 2001).

Another fundamental component to an integrated lab chip design is the on-line ionization source interface. In particular, the nano-electrospray ionization (nESI) source is the most suitable interface for lab chip designs when MS-based platforms are used as the detection system. Contributing factors for the ideality of the nESI source include their intrinsic non-destructive operation leading to the efficient ionization of a broad range of biomolecules including sugars, amino acids, fatty acids, nucleic acids, peptides and proteins. Therefore, the chemical integrity of these biomolecules remains intact thanks to this "soft" ionization imparted by the nESI interface (Wilm, 2011). Another contributing factor is that the nESI efficiency can be enhanced at the low nL/mL flow rate regime, provided of course that the correct geometry is utilized (Figure 10). In fact, at these flow rates, the nESI interface is less prone to the suppression effects observed when reagents essential to the operation of capillary electrophoresis and electrochromatography.

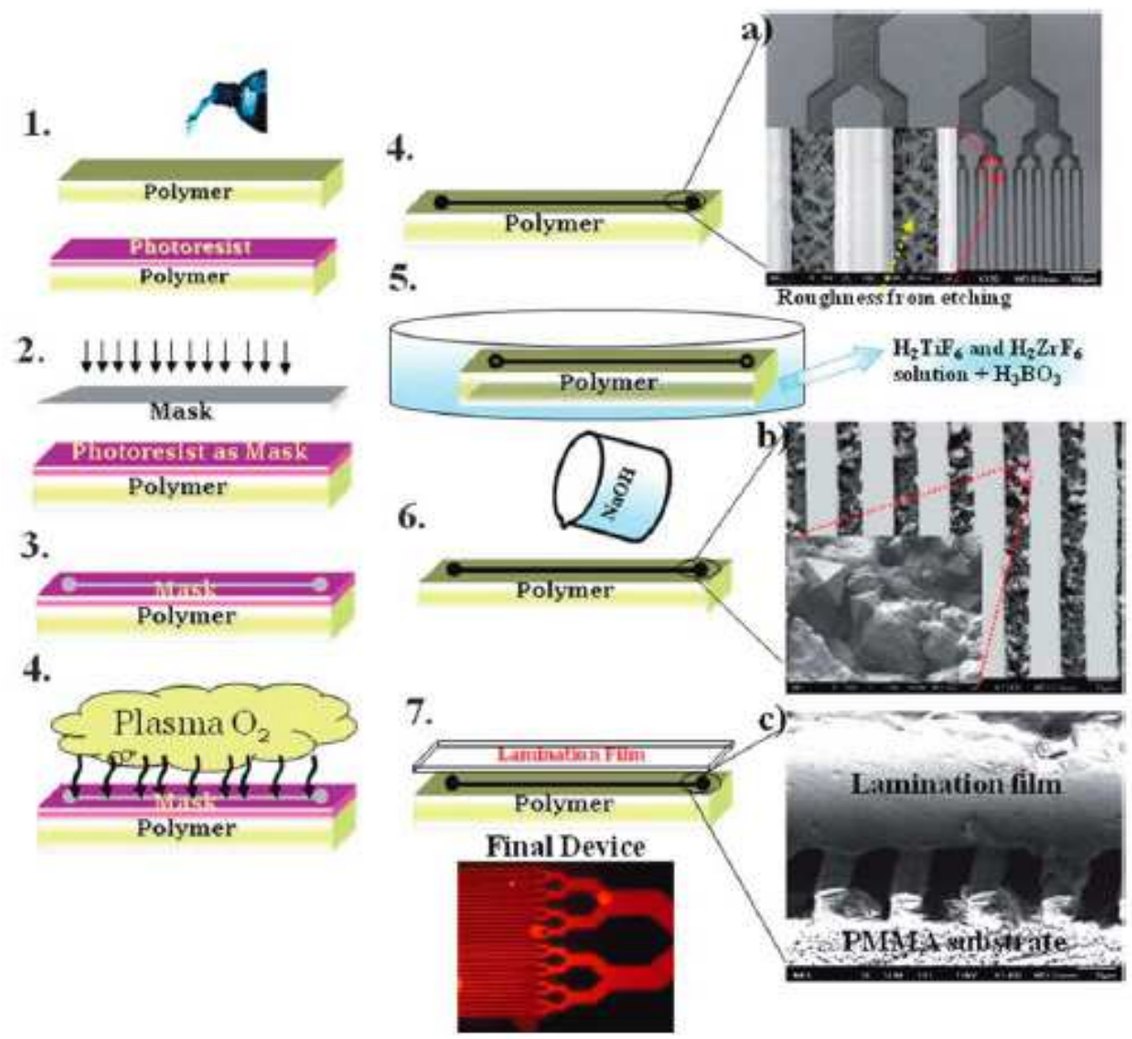

Fig. 6. Schematic representation of the fabrication process with direct lithography and plasma etching followed by liquid deposition of the TiO_2–ZrO_2 stationary phase: (1) spin coating of a thin inorganic (ORMOCER) photoresist as an etching mask on PMMA sheets, (2) lithography on photoresist polymer, using mask exposure, (3) photoresist development, (4) deep plasma etching of polymeric substrate, (5) liquid deposition of the thin TiO2–ZrO2 film and baking at 95°C, (6) rinsing with 0.1 M NaOH and DI water and baking at 95°C, and (7) sealing with lamination films. The relative thickness in the figure does not correspond to the real thickness. SEM image insets: (a) a PMMA micro-column consisting of 32 parallel microchannels after etching (a zoomed image showing the roughness at the microchannel bottom is also shown), (b) a PMMA micro-column after liquid phase deposition of TiO_2–ZrO_2 (a zoomed image of the crystallites is also shown), and (c) a cross-section of the column, after bonding with the lamination film (for details see also ESI†) (Tsougeni et al., 2011).

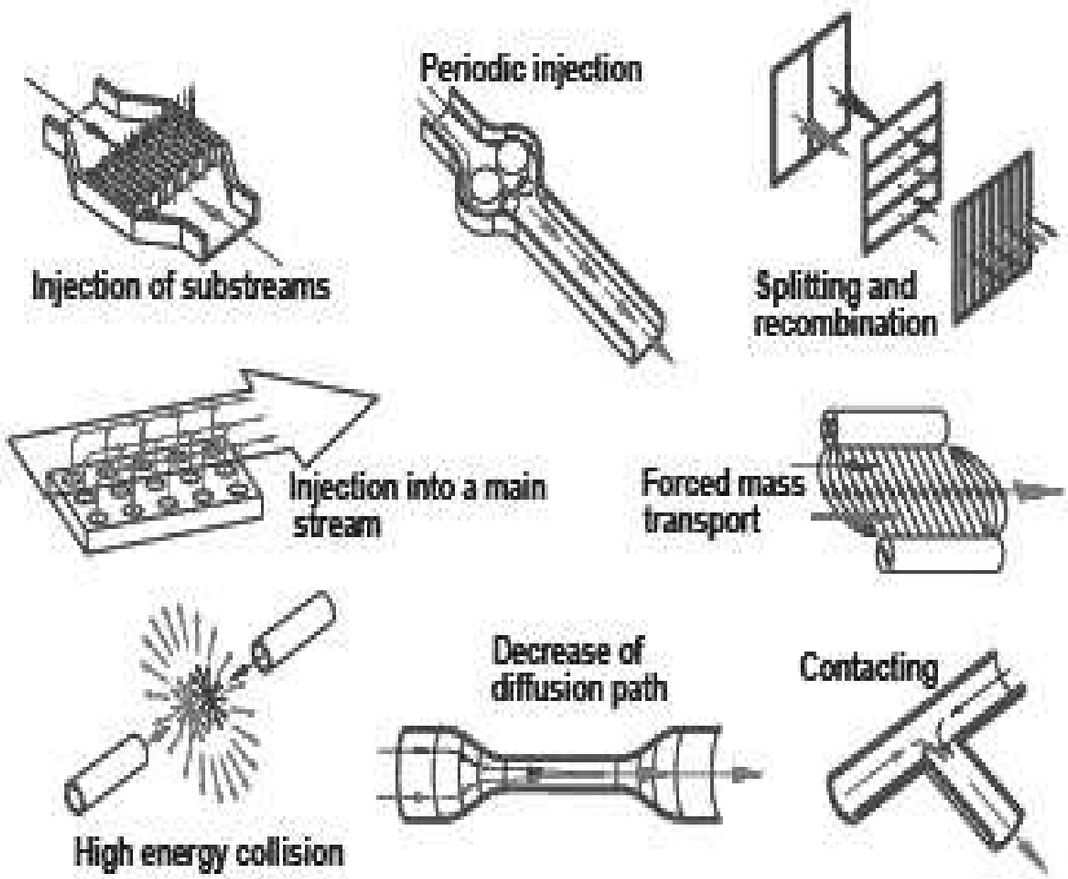

Fig. 7. Illustrative representations of various mixing chambers with different operational modes that are applicable to lab chip devices. These designs allow for the efficient mixing of reagents at nano-flow rates and assist the integration of various modes of chromatographic technique (i.e. multidimensional MudPIT) (Jahnisch et al., 2004).

Such integrated lab chip designs will allow for the effective miniaturization and automation of multi-dimensional MudPIT approaches illustrated in the previous section. Theoretically, such a lab chip reconfiguration of the more traditional lab bench analytical methodology can increase the bioanalyte sensitivity by more than several orders of magnitude. Consequently, full proteomes can be fully characterized by vastly smaller biological starting amounts (i.e. fg levels vs. µg levels). At this level of analytical sensitivity techniques such as laser capture microdissection and cell sorting can effectively be incorporated to research protocols. Also biomolecules constituting exosome entities found in plasma occurring at very low levels can also be detected. It is hypothesized that exosome biology may help explain how a particular organ secrete or shed proteins and other biomolecules such as DNA and mRNA into the systemic circulation. The exosome composition may be highly depended on the disease state of the organ (i.e. initiation stage carcinogenesis). Therefore exosomes may play a crucial role in using plasma as a biopsy source to interrogate the tissue pathophysiology status.

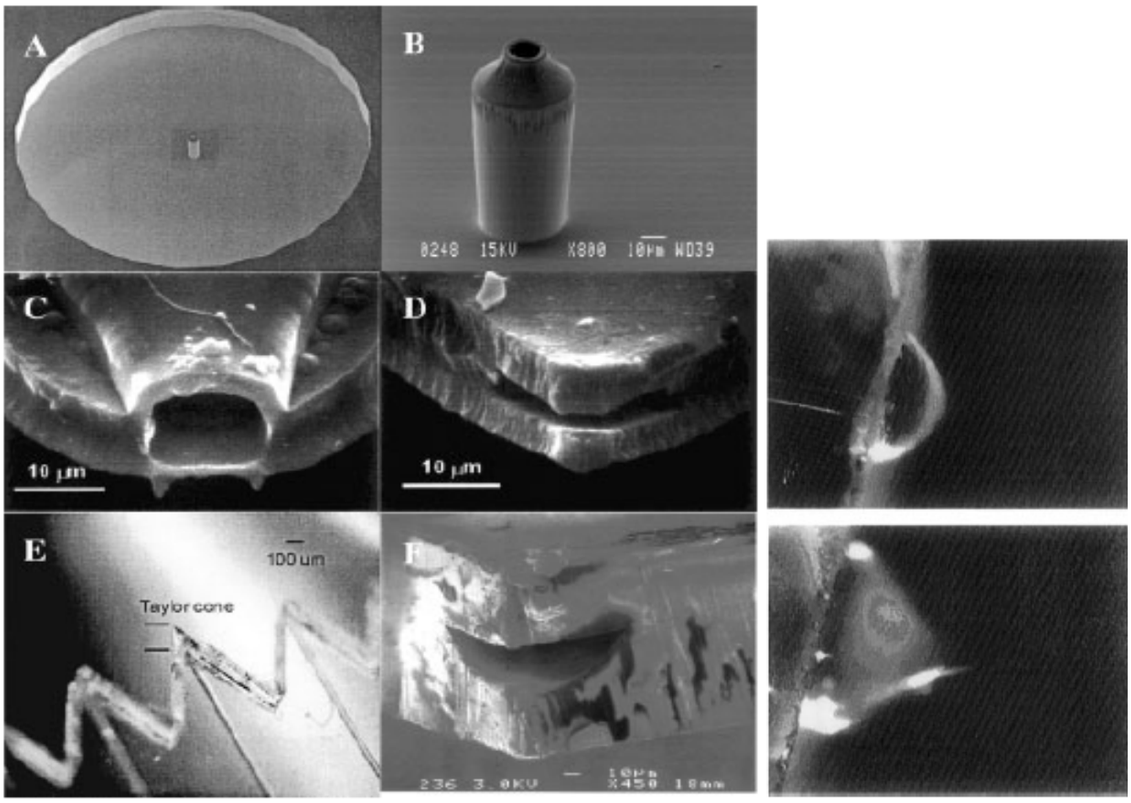

Fig. 8. Schematic nESI designs that allow its operation at the low nL/mL flow regime. Geometries, dimensions, along with their material compositions all play a pivotal role in the optimal nESI process (Lion et al., 2003).

6. Conclusion

The molecular characterization, dissection and appreciation of carcinogenesis is, undoubtedly, much more complex than we ever envisaged. The disease of cancer *per se* remains complicated, unpredictable and multifaceted – either by the impact and influence of genes, the environment, behaviour, proteins or all combined (epigenetics). In this chapter we have discussed the solid tumour of prostate cancer, a disease which falls in to two classes – indolent or aggressive. Pathologically, via immunohistology the disease 'looks' very different but at early and intermediate stages, clinically, we find it difficult to differentiate benign from malignant. We struggle to decide which glands can be left and monitored versus which that need resection and immediate therapy. The influence of 'omics and especially proteomics now has the power to categorize prostate cancer – not just the disease itself but possibly those men who may be prone to developing prostate cancer, especially the aggressive form and identify those men who need immediate intervention (Larkin et al., 2011). The next decade will see huge strides in stratifying 'normo' physiology and disease and we have presented a wealth of information here which helps to explain how the state-of-the-art methodologies and excellent clinical and patient stratification we currently have will enable this.

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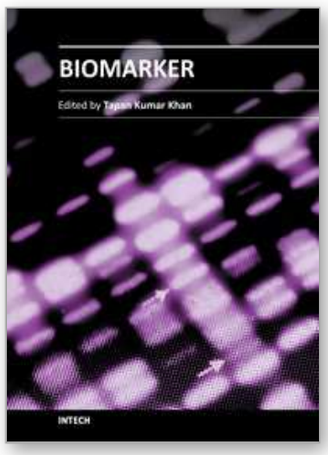

Biomarker

Edited by Prof. Tapan Khan

ISBN 978-953-51-0577-0
Hard cover, 392 pages
Publisher InTech
Published online 27, April, 2012
Published in print edition April, 2012

Clinicians, scientists, and health care professionals use biomarkers or biological markers as a measure of a person's present health condition or response to interventions. An ideal -biomarker should have the following criteria: (I) ability to detect fundamental features of the disease, (II) ability to differentiate from other closely related diseases, (III) ability to detect early stages and stages of progression, (IV) the method should be highly reliable, easy to perform and inexpensive, and (V) sample sources should be easily accessible from body. Most of the chapters in this book follow the basic principle of biomarkers.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Spiros D. Garbis and Paul A. Townsend (2012). The Discovery of Cancer Tissue Specific Proteins in Serum: Case Studies on Prostate Cancer, Biomarker, Prof. Tapan Khan (Ed.), ISBN: 978-953-51-0577-0, InTech, Available from: http://www.intechopen.com/books/biomarker/the-discovery-of-cancer-tissue-specific-proteins-in-serum-case-studies-on-prostate-and-breast-cance

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